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Multiple origins of hydrogenosomes: functional and phylogenetic evidence from the ADP/ATP carrier of the anaerobic chytrid *Neocallimastix* sp.

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Summary

A mitochondrial-type ADP/ATP carrier (AAC) has been identified in the hydrogenosomes of the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2. Biochemical and immunocytochemical studies revealed that this ADP/ATP carrier is an integral component of hydrogenosomal membranes. Expression of the corresponding cDNA in *Escherichia coli* confers the ability on the bacterial host to incorporate ADP at significantly higher rates than ATP – similar to isolated mitochondria of yeast and animals. Phylogenetic analysis of this AAC gene (*hdgaac*) confirmed with high statistical support that the hydrogenosomal ADP/ATP carrier of *Neocallimastix* sp. L2 belongs to the family of veritable mitochondrial-type AACs. Hydrogenosome-bearing anaerobic ciliates possess clearly distinct mitochondrial-type AACs, whereas the potential hydrogenosomal carrier Hmp31 of the anaerobic flagellate *Trichomonas vaginalis* and its homologue from *Trichomonas gallinae* do not belong to this family of proteins. Also, phylogenetic analysis of genes encoding mitochondrial-type chaperonin 60 proteins (HSP 60) supports the conclusion that the hydrogenosomes of anaerobic chytrids and anaerobic ciliates had independent origins, although both of them arose from mitochondria.

Introduction

Certain unicellular anaerobes such as, for example, the parabasalian flagellate *Trichomonas*, the amoeboflagellate *Psalteriomonas lanterna*¸ the ciliates *Trimyema compressum*, *Plagiopyla nasuta*, *Dasytricha ruminantium*, *Nyctotherus ovalis* and the chytridiomycete fungi *Neocallimastix* sp. and *Piromyces* sp. possess 'hydrogenosomes' instead of mitochondria (Vogels *et al*., 1980; Yarlett *et al*., 1981; 1983; 1986; van Bruggen *et al*., 1983; Zwart *et al*., 1988; Broers *et al*., 1990; Gijzen *et al*., 1991; Marvin-Sikkema *et al*., 1992; 1993a; reviewed by Müller, 1993; Fenchel and Finlay, 1995; Hackstein *et al*., 1999; 2001; Roger, 1999). Hydrogenosomes are membrane-bound organelles that compartmentalize terminal reactions of the eukaryotic energy metabolism. However, unlike mitochondria, which fulfil this function in aerobic eukaryotes, hydrogenosomes are found exclusively in unicellular anaerobes. Hydrogenosomes generate hydrogen, acetate (or acetate and formate respectively) and carbon dioxide because they can use protons as an electron acceptor (Müller, 1993; 1998). Despite the obvious differences from the mitochondrial metabolism and despite their occurrence in only distantly related taxa of anaerobic protists, a wealth of (circumstantial) evidence argues for a common ancestry of mitochondria and hydrogenosomes (Embley *et al*., 1997; Martin and Müller, 1998; Plümper *et al*., 1998; 2000; Andersson and Kurland, 1999; Hackstein *et al*., 1999; Dyall and Johnson, 2000;

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Rotte *et al*., 2000). However, the available data still cannot provide a simple answer to the question whether all hydrogenosomes are the same or, more explicitly, are hydrogenosomes varieties of 'anaerobic' mitochondria or convergent adaptations of different cellular compartments (or endosymbionts) to life under anoxic conditions?

Remarkably, the hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis* look like mitochondria and recently we have provided evidence for the presence of a mitochondrial-type genome in these organelles (Akhmanova *et al*., 1998a; van Hoek *et al*., 2000). All available data suggest that the hydrogenosomes of *N. ovalis* represent a kind of anaerobic mitochondria that shares a common ancestry with the veritable mitochondria of aerobic ciliates (van Hoek *et al*., 2000). In contrast, the prototypical hydrogenosomes of *Trichomonas* spp. exhibit only a weak morphological similarity to mitochondria and, importantly, they lack any genome that could provide unequivocal evidence for their ancestry (Benchimol *et al*., 1996; Clemens and Johnson, 2000). Also, the stacked hydrogenosomes of *Psalteriomonas* (Broers *et al*., 1990) and the elusive hydrogenosomes of *Neocallimastix* (Marvin-Sikkema *et al*., 1992; 1993a) do not resemble mitochondria. These hydrogenosomes lack a genome (Palmer, 1997; van der Giezen *et al*., 1997; J. H. P. Hackstein, unpublished), and it is impossible to validate the mitochondrial ancestry of these organelles directly. Furthermore, phylogenetic analysis of several nuclear-encoded hydrogenosomal proteins failed to provide straightforward evidence for a mitochondrial ancestry. Rather, it revealed a mosaic of mitochondrial and non-mitochondrial ancestries (Bui *et al*., 1996; Germot *et al*., 1996; Akhmanova *et al*., 1998a; Hackstein *et al*., 1999; Horner *et al*., 1999; 2000; Voncken *et al*., 2002).

Because of their unique properties, hydrogenosomal ADP/ATP carriers might allow an unequivocal spotting of the ancestries of those hydrogenosomes that lack a genome (Andersson and Kurland, 1999; Emelyanov, 2001). ADP/ATP carriers are essential for the function of energy-generating organelles such as mitochondria and hydrogenosomes, and it is unlikely that they have been subject to lateral gene transfer in the course of their evolution because they are detrimental for every autonomous free-living or parasitic/endosymbiotic organism. Mitochondria, for example, have evolved a well-characterized family of unique ADP/ATP carriers (AACs) that facilitate the import of ADP and the export of ATP (Aquila *et al*., 1987; Klingenberg, 1989; 1992; Palmieri, 1994; Palmieri *et al*., 2000). Nucleotide carriers of hydrogenosomes have not yet been identified, although P. J. Johnson and col-

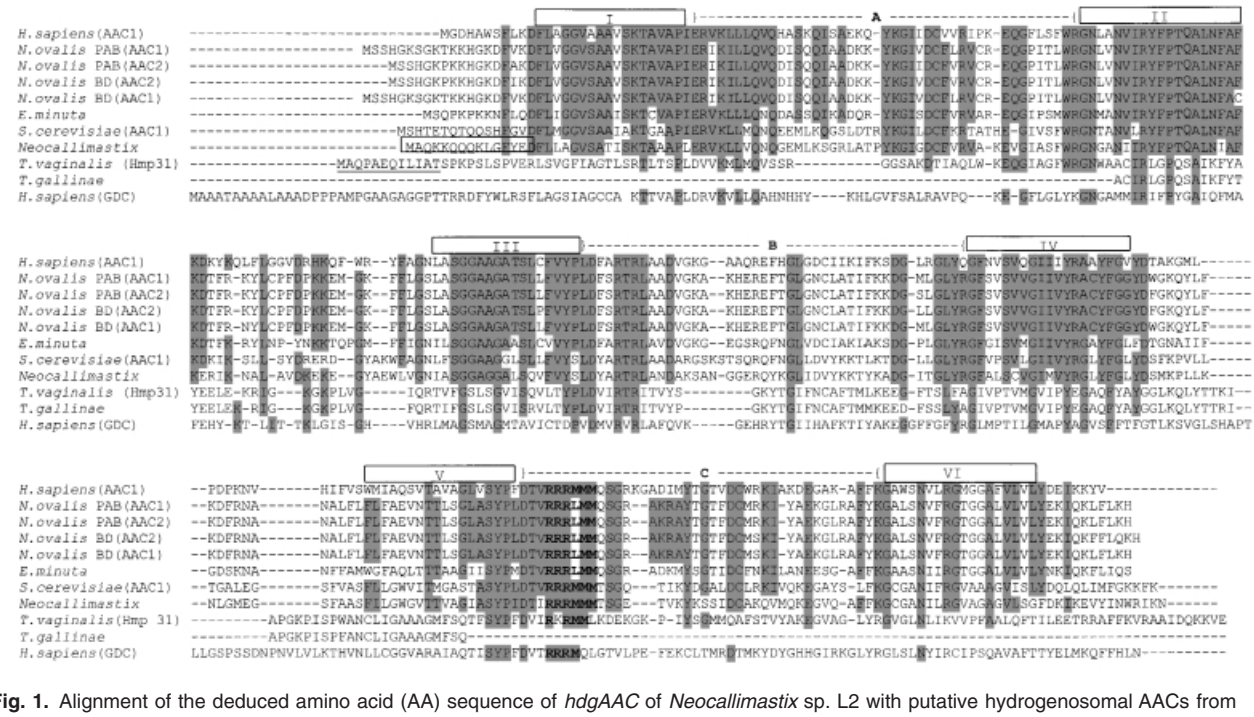

Fig. 1. Alignment of the deduced amino acid (AA) sequence of *hdgAAC* of *Neocallimastix* sp. L2 with putative hydrogenosomal AACs from anaerobic ciliates, Hmp31 of *Trichomonas*, the human Grave's disease protein and representative mitochondrial AACs from aerobic eukaryotes (*Homo sapiens*, *Saccharomyces cerevisiae* and *Euplotes minuta*). *N. ovalis* PAB, anaerobic ciliate from *Periplaneta americana* strain Bayer; *N. ovalis* BD, *Nyctotherus* from *Blaberus fuscus* strain Düsseldorf (cf. van Hoek *et al*., 1998). *Euplotes minuta* is an aerobic, mitochondriate ciliate. The letters A, B and C indicate the three-domain structure that is characteristic of mitochondrial AACs. The six hydrophobic, membrane-spanning regions are indicated as boxes (I–VI). The 'RRRMMM' motif is printed in bold. Gaps are indicated by dashes. The 15 N-terminal amino acids of the *Neocallimastix* AAC that have been confirmed by protein sequencing are boxed. The (cleaved) presequence of Hmp31 of *T. vaginalis* is underlined.

laborators described an abundant protein (Hmp31) from hydrogenosomal membranes of the parabasalian flagellate *Trichomonas vaginalis* that might be a representative of hydrogenosomal nucleotide carriers (Dyall *et al*., 2000). Notably, phylogenetic analysis of the gene encoding Hmp31 identified it as a member of the large gene family that encodes the various mitochondrial carrier proteins (cf. El Moualij *et al*., 1997; Nelson *et al*., 1998; Palmieri *et al*., 2000). However, Hmp31 did not cluster with veritable mitochondrial ADP/ATP carriers, and the function of Hmp31 has remained elusive until now.

On the other hand, functional evidence for the existence of a mitochondrial-type AAC in the hydrogenosomes of the anaerobic chytrid *Neocallimastix* sp. L2 was provided several years ago (Marvin-Sikkema *et al*., 1994). Treatment of isolated hydrogenosomes with bongkrekic acid and carboxyatractylate, well-characterized inhibitors of mitochondrial AACs (Winkler and Neuhaus, 1999), inhibited the hydrogen production of the organelles by about 75%. As molecular analysis was out of the scope of this study, purification and characterization of the target protein has not been attempted, and the gene encoding the putative mitochondrial-type AAC remains unknown too. Here, we describe the identification of a mitochondrial-type ADP/ATP carrier in the hydrogenosomes of *Neocallimastix* sp. L2. This protein is encoded by a gene that shares a high level of sequence identity with the mitochondrial-type AACs of yeasts, fungi and plants. By functional expression in *Escherichia coli*, we provide evidence that this hydrogenosomal protein transports ADP and ATP. Phylogenetic analysis also confirms that anaerobic ciliates possess mitochondrial-type AACs, which are quite distinct from the fungal ones. We conclude that the hydrogenosomes of anaerobic chytrids evolved from fungal mitochondria, whereas the hydrogenosomes of anaerobic ciliates evolved from ciliate mitochondria.

Results

Molecular genetic characterization of a putative hydrogenosomal ADP/ATP carrier

Using polymerase chain reaction (PCR) and the rapid amplification of cDNA ends (RACE) procedure, we isolated a 1051 bp cDNA ('*hdgaac*') from *Neocallimastix* sp. L2. DNA sequence analysis revealed the presence of a complete open reading frame (ORF) of 924 bp (accession no. AF340168). The deduced amino acid sequence (hdgAAC) exhibits high similarity (42–59% identity, 57–77% similarity) to the sequences of well-characterized mitochondrial ADP/ATP carriers from the various aerobic eukaryotes. The sequence predicts a protein with an apparent molecular weight of 32 kDa, which is well within the expected size range of the AAC proteins (Palmieri, 1994). The putative protein exhibits the characteristic tri-repeat structure that is observed in all mitochondrial AACs analysed so far (Saraste and Walker, 1982; Aquila *et al*., 1987; Klingenberg, 1989; 1992; Palmieri, 1994). The clusters of charged residues are conserved. Moreover, adjacent to the hydrophobic region V, the motif RRRMMM can be identified, which seems to be conserved among veritable mitochondrial ADP/ATP carriers from yeast to man (Fig. 1). Notably, this motif is changed to RRRLMM in the putative AACs from both the aerobic (*Euplotes*) and anaerobic (*Nyctotherus*) ciliates. The putative ciliate AACs were isolated by PCR using genomic DNA as template.

Southern blotting using *Neocallimastix* DNA argues for the presence of only one gene with high similarity to the *hdgaac* probe (Fig. 2A). Also, Northern blotting does not provide evidence for the expression of more than one *hdgaac* gene: hybridization of poly(A)$^+$ RNA with the *hdgaac* probe revealed the presence of a single transcript (Fig. 2B). Its length (1750 nucleotides) exceeds the length of the identified ORF (924 bp), but not the length of the genomic *Eco*RI and *Xba*I fragments. These enzymes cut outside the ORF. The length difference between the ORF and the primary transcript is most probably caused by the presence of AT-rich 5′ and 3′ untranslated regions, which are characteristic of all *Neocallimastix* and *Piromyces* cDNAs analysed so far (Reymond *et al*., 1992; Durand *et al*., 1995; Fanutti *et al*., 1995; Akhmanova *et al*., 1998b; 1999; Voncken *et al*., 2002).

Phylogenetic analysis

Phylogenetic analysis shows that the deduced hdgAAC sequence of *Neocallimastix* sp. L2 clusters with the mito-

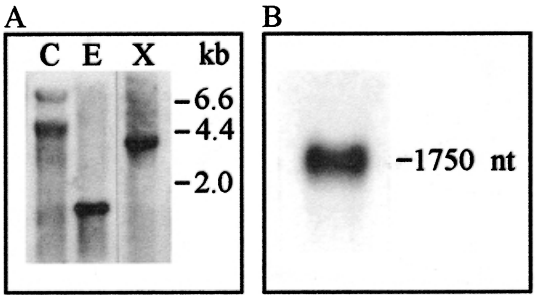

Fig. 2. Southern and Northern blots of the *hdgaac* of *Neocallimastix* sp. L2.
A. Genomic DNA (10 μg) of *Neocallimastix* sp. L2 was digested with the restriction enzymes *Cla*I (C), *Eco*RI (E) and *Xba*I (X). The enzymes *Eco*RI and *Xba*I do not cut inside the coding region, whereas the enzyme *Cla*I has a single recognition site in the coding region. Consistent with this fact, the Southern blots with genomic DNA digested with *Eco*RI and *Xba*I reveal only a single hybridizing band that is larger than the transcript. Probing with *hdgaac*, labelled with [α-^{32}P]-dATP, does not provide evidence for more than one genomic copy of *hdgaac*.
B. Northern blot analysis of 5 μg of poly(A)$^+$ RNA reveals the presence of a single transcript.

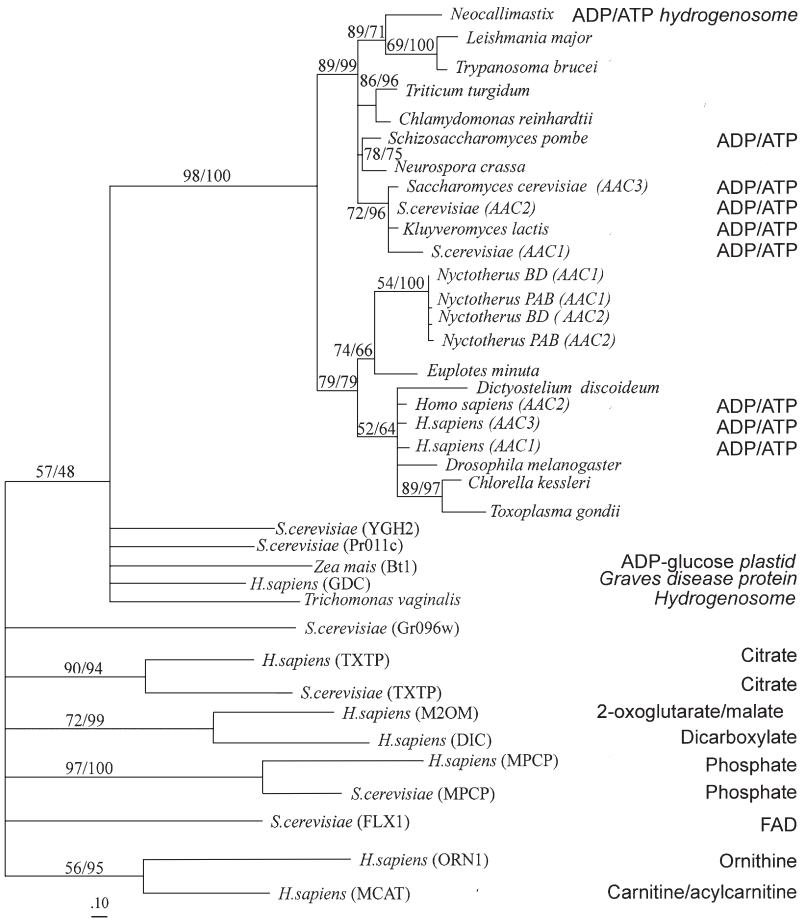

Fig. 3. Phylogenetic tree of representatives of the mitochondrial carrier family. Phylogenetic trees were constructed using the neighbour-joining (NJ; Saitou and Nei, 1987) and quartet-puzzling algorithms (Strimmer and von Haeseler, 1996). The first number indicates the PUZZLE reliability values, the second number the NJ bootstraps. At the right, experimentally determined substrate specificities of the carriers or other relevant features are indicated (the latter in italics). In contrast to the single (hydrogenosomal) AAC of *Neocallimastix*, several AAC isoforms exist in the anaerobic ciliates: *Nyctotherus* PAB, *N. ovalis* from *Periplaneta americana* var. Bayer; *Nyctotherus* BD, *N. ovalis* from *Blaberus fuscus* var. Düsseldorf (cf. van Hoek *et al*., 1998). *Euplotes minuta* is an aerobic, mitochondriate ciliate. For the SWISSPROT names or GenBank identifiers of the sequences used in the tree, see *Experimental procedures*. Bar, evolutionary distance.

chondrial AACs of yeasts, fungi, plants and the parasites *Leishmania major* and *Trypanosoma brucei* (Fig. 3). This cluster is clearly distinct from that encompassing the putative hydrogenosomal AACs of *Nyctotherus* and the mitochondrial AACs of protozoa (e.g. *Euplotes*), animals and man. Notably, not only all the veritable mitochondrial AACs, but also the hydrogenosomal AACs of *Neocallimastix* sp. L2 and the various (putative) AACs of the *Nyctotherus* species are clearly different from the hydrogenosomal protein Hmp31 of *T. vaginalis* and its homologue from *T. gallinae* (Figs 1 and 3; cf. Dyall *et al*., 2000). As the PCR strategy, which was successful in cloning a broad spectrum of protist AACs (i.e. *Nyctotherus*, *Euplotes*, *Tetrahymena*, *Toxoplasma* and *Neocallimastix*), failed to amplify homologous genes from both *T. vaginalis* and *T. gallinae*, it is likely that potential mitochondria-like AACs from *Trichomonas* must be rather divergent, if present at all.

Functional expression in E. coli

The complete ORF encoding the putative hydrogenosomal AAC of *Neocallimastix* sp. L2 has been cloned in the plasmid pET16b and expressed in the *E. coli* strains BL-21 and C43. After induction, both strains exhibited a substantial uptake of [α-^{32}P]-ADP (Table 1). An apparent K_m of 165 $\pm$ 13.2 μM (n = 3) for the uptake of ADP has been determined. Incubation with [α-^{32}P]-ATP resulted in a much lower uptake of radioactivity, in clear contrast to the rapid uptake of [α-^{32}P]-ATP by transgenic strains of *E. coli* expressing plastidic or *Rickettsial* ATP/ADP transporters (Tjaden *et al*., 1998). As control cells did not import either [α-^{32}P]-ADP or [α-^{32}P]-ATP at significant rates (Table 1), it must be concluded that: (i) the recombinant *hdgAAC* from *Neocallimastix* sp. L2 is functionally expressed in *E. coli*; (ii) the recombinant protein is inserted into the bacterial membrane; and (iii) the hdgAAC from *Neocallimastix* sp. L2 is a functional AAC that imports ADP. Table 1 shows that the *hdgaac* gene product is capable of both ATP and ADP uptake. The substrate specificity is limited to ADP and ATP (Table 2), and preliminary results from back-exchange experiments suggest an export of ATP, which must be facilitated by the recombinant hdgAAC (not shown). These data are in agreement with results that were obtained by other groups with isolated, intact mitochondria: ADP is also imported preferentially in mitochondria. An electrogenic gradient (positive outside/negative inside) seems to be responsible for the

Table 1. Uptake of radioactively labelled adenine nucleotides into *E. coli* expressing *hdgaac.*

Substrate	Rate of transport (nmol mg^{-1} protein h^{-1})		
	E. coli-pET16b (control, 50 μM)	*E. coli*-pIH6 (50 μM)	*E. coli*-pIH6 (500 μM)
[α-^{32}P]-ATP	0.06 ± 0.01	0.84 ± 0.08	3.46 ± 0.45
[α-^{32}P]-ADP	0.07 ± 0.01	3.96 ± 0.48	12.11 ± 0.81

Radioactively labelled compounds were present at a final concentration of 50 μM for the control cells (*E. coli*-pET16b) and at a final concentration of 50 μM or 500 μM for the *E. coli* cells (pIH6), which are harbouring the plasmid including the ADP/ATP carrier gene (*hdgaac*). Incubation was carried out with IPTG-induced *E. coli* cells for 4 min. Uptake was terminated by rapid filtration of *E. coli* cells. Data are the mean of three independent experiments.

fact that the import of ADP^{3-} by far exceeds that of ATP^{4-} (cf. Klingenberg, 1989).

Localization of the gene product

Homogenates of *Neocallimastix* sp. L2 were subjected to differential centrifugation in order to obtain a 30 000 *g* organelle fraction that is enriched in hydrogenosomes (Marvin-Sikkema *et al.*, 1993a). Western blotting using the organelle fraction and a heterologous antiserum directed against mitochondrial AACs of *Saccharomyces cerevisiae* revealed a cross-reacting membrane protein of 32 kDa. This band was absent from the cytosolic fraction (Fig. 4A and B). The cross-reacting band was excised from the polyvinylidene difluoride (PVDF) membrane and subjected to amino-terminal protein sequencing. The first 15 N-terminal amino acids of the protein appeared to be identical to the first 15 deduced amino acids of the cloned *hdgaac* gene (Fig. 1), confirming that the authentic AAC protein was recognized by the antiserum. In order to analyse whether the cross-reacting protein was membrane associated or an integral component of the hydrogenosomal membranes, the proteins of the hydrogenosomal fraction were subjected to proteinase K treatment (Fig. 4C) and salt extraction procedures (Fig.

Table 2. Effects of various metabolites on [α-^{32}P]-ADP uptake into IPTG-induced *E. coli* cells expressing *hdgaac.*

Effector	Rate of ADP transport	
	(nmol mg^{-1} protein h^{-1})	(%) of Control
None	3.09	100.0
ADP	0.21	6.8
ATP	1.51	48.8
AMP	2.98	96.4
UMP	3.29	106.4
UTP	3.23	104.5
CTP	3.09	100.0
GTP	3.24	104.8
dATP	2.73	88.3
dTTP	3.03	98.1
dCTP	3.08	99.7
dGTP	2.82	91.3
UDPGal	2.91	94.2
UDPGlc	2.78	90.0
NADH	3.19	103.4

The uptake of [α-^{32}P]-ADP in competition with various effectors was measured as described in Table 1. The various effectors were given at a concentration of 300 μM. [α-^{32}P]-ADP was present at a concentration of 100 μM. Uptake was carried out for 4 min and stopped by rapid filtration. Data are the mean of three independent experiments. SE <8% of the mean values.

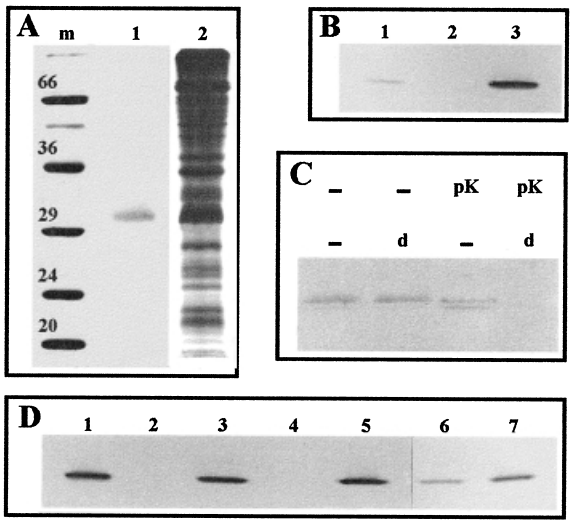

Fig. 4. Western blot analysis and subcellular localization of hdgAAC.
A. After SDS–PAGE, a single band of the membrane fraction of isolated hydrogenosomes reacts with heterologous antiserum directed against a mitochondrial AAC from yeast (lane 1). Lane 2, Coomassie brilliant blue-stained membrane proteins. m, marker proteins.
B. The cross-reacting protein is highly enriched in the hydrogenosomal fraction (lane 3) and absent from the cytosolic fraction (lane 2). Lane 1, homogenate of the fungal mycelium.
C. Protection assays localizing hdgAAC in the hydrogenosomes: only after incubation with proteinase K (pK) and detergent (d) is the hdgAAC digested completely. d, Triton X-100/deoxycholate; (–), no additions of proteinase K or detergent.
D. Differential extraction of hdgAAC from the (lysed) hydrogenosomal fraction. Lane 1, total homogenate; lane 2, supernatant, hydrogenosomal matrix proteins; lane 3, pellet of lysed hydrogenosomes; lane 4, supernatant, membrane-associated, 1 M KCl-soluble proteins; lane 5, pellet after high salt (1 M KCl) extraction; lane 6, supernatant of 0.1 M carbonate, pH 11.5, extractable proteins; 7, integral membrane proteins, after two extractions with 0.1 M carbonate. All data confirm localization of hdgAAC in the hydrogenosomal membranes. The extractable fraction of AAC is significant. This might result from the lack of cardiolipin in *Neocallimastix* sp. L2 (see Table 3 and *Supplementary material*).

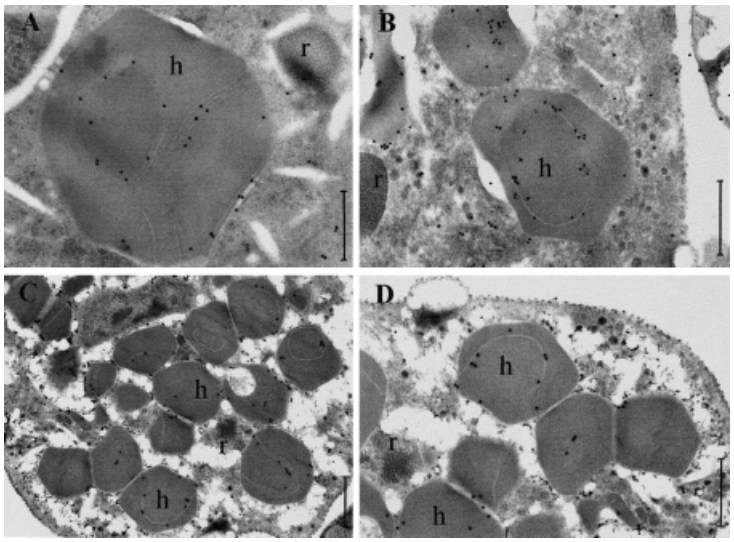

Fig. 5. Immunocytochemical localization of hdgAAC in the hydrogenosomes of *Neocallimastix* sp. L2. Mycelia and zoospores from *Neocallimastix* sp. L2 were fixed, embedded and ultrathin sectioned. After immunogold labelling with the heterologous anti-AAC serum, indicating the presence of hdgAAC, the labelling is confined to the hydrogenosomal membranes, both the internal and external ones. h, hydrogenosomes; r, ribosome globules (Munn *et al*., 1988). Bars represent 0.5 μm.

4D). Incubation of the pellet fraction with 1 M KCl failed to release any AAC protein, excluding the possibility that the hydrogenosomal AAC was a membrane-associated or peripheral protein. After carbonate treatment, the majority of the protein was retained in the pellet fraction (Fig. 4D). Moreover, the protein was also largely protected against proteinase K digestion in the absence of detergent. Under these conditions, proteinase K could only remove a small part of the protein (Fig. 4C). However, AAC disappeared completely from the organelle fraction when both detergent and proteinase K were added (Fig. 4C). These observations led to the conclusion that hdgAAC is an integral constituent of the hydrogenosomal membranes.

Immunocytochemistry

Immunogold labelling of ultrathin sections from zoospores of *Neocallimastix* sp. L2 revealed that the anti-AAC labelling was confined to the hydrogenosomal membranes, not only to the inner, but also to the bounding ones (Fig. 5). Because this seemed rather unusual, we

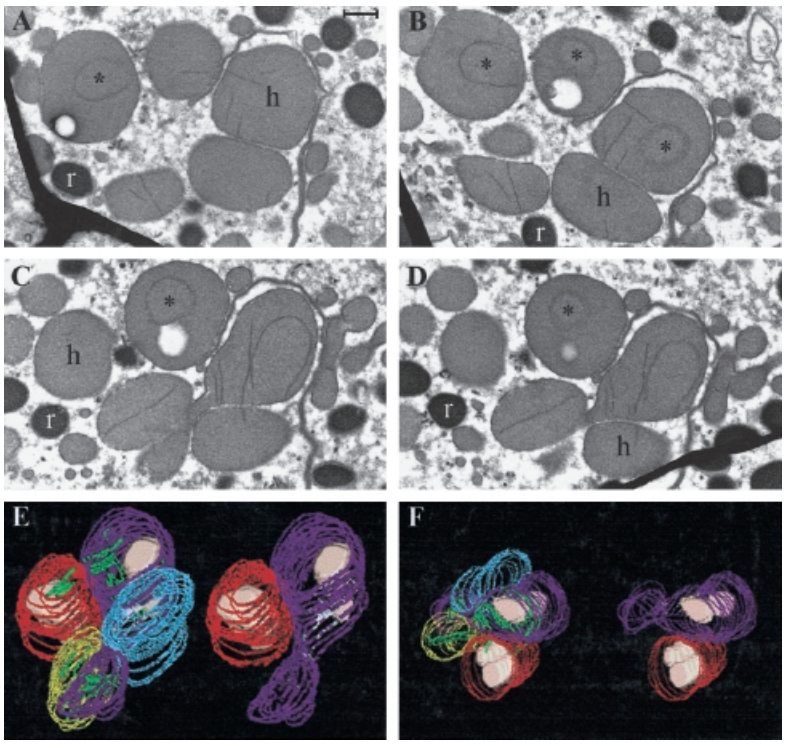

Fig. 6. Serial sectioning and three-dimensional reconstruction of the hydrogenosomes of *Neocallimastix* sp. L2.
A–D. Electron micrographs of four of the 16 serial sections of zoospores that were used for the three-dimensional reconstruction shown in (E) and (F). Bar = 0.5 μm; h, hydrogenosomes; r, ribosome globules (Munn *et al*., 1988). Asterisk, internal vesicular structures.
E and F. Computer-aided three-dimensional reconstruction of a group of hydrogenosomes, viewed from different angles. The reconstruction reveals that vesicles (labelled in pink) are present in the matrix of most of the hydrogenosomes, confirming that the hydrogenosomes of zoospores and hyphae are not fundamentally different. Moreover, the reconstruction confirms that the morphology of isolated hydrogenosomes is similar to the situation *in vivo*, albeit the space between the membranes of the internal vesicles and the bounding membranes is greatly reduced after isolation in (hypertonic) isolation medium.

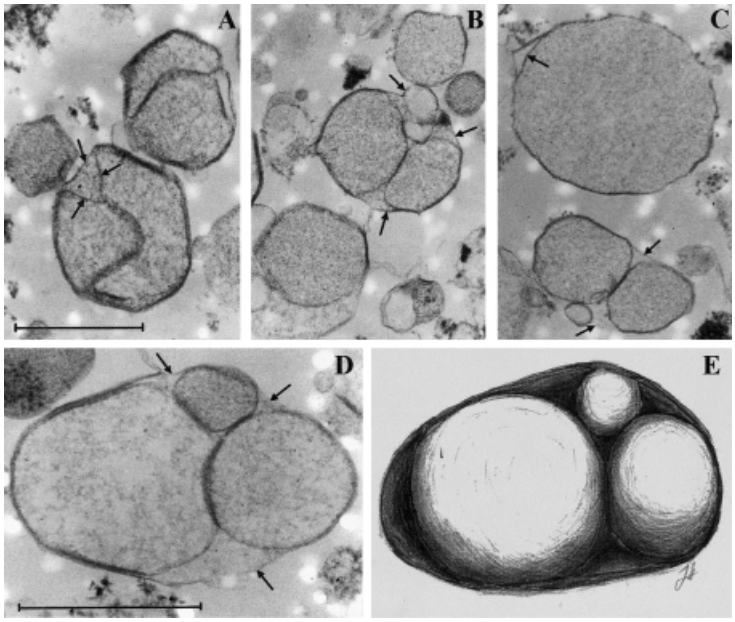

Fig. 7. Hydrogenosomes obtained by cell fractionation.
A–C. Osmotic treatment of isolated hydrogenosomes with solutions of 1.0 M (A), 0.5 M (B) and 0.0 M (C) sucrose.
Bar = 0.5 μm.
D. Median section of a hydrogenosome obtained after cellular fractionation.
Bar = 0.5 μm.
E. An artist's view of the hydrogenosome shown in (D). Arrows indicate 'single' membranes.

analysed the structure of the hydrogenosomes in more detail. Serial sectioning followed by computer-aided three-dimensional reconstruction revealed that most of the internal membrane structures belonged to vesicles (Fig. 6). Up to three vesicles were found inside the hydrogenosomes, both in the hydrogenosomes of zoospores fixed in the culture medium and in the isolated hydrogenosomes, which were obtained after cellular fractionation of mycelial homogenates (Fig. 7). In order to determine whether a double membrane or only a single membrane bounded the organelle, isolated hydrogenosomes were subjected to osmotic treatment in an anaerobic isolation buffer containing 0.0, 0.1, 0.25, 0.5 and 1.0 M sucrose. Figure 7 shows that the organelles are sensitive to the osmotic treatment. Hypertonic treatment resulted in a close opposition of the inner and outer membranes, whereas hypotonic treatment eventually caused a rupture of the outer membrane. This membrane and also the inner membranes are single membranes. Both the outer and the inner membranes are morphologically indistinguishable, in clear contrast to the hydrogenosomal membranes of *N. ovalis* and the membranes of veritable mitochondria (cf. Fig. 1 in Akhmanova *et al*., 1998a).

Also, the chemical composition of the various hydrogenosomes is different: mass spectroscopy revealed that the hydrogenosomal membranes of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 are devoid of cardiolipin, whereas the hydrogenosomes of the anaerobic ciliate *N. ovalis* possess cardiolipin (Table 3).

Discussion

We have shown that a mitochondrial-type ADP/ATP carrier forms an integral component of hydrogenosomal membranes of the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2. The DNA sequence of the corresponding *hdgaac* gene exhibits the characteristics of a mitochondrial AAC, e.g. a tripartite structure of $\approx 3 \times 100$ amino acids, six membrane-spanning domains, conserved amino acids and the 'RRRMMM' motif, which is shared by many mitochondrial AACs from yeast to man (Fig. 1; Klingenberg, 1989; 1992; Palmieri, 1994). Phylogenetic analysis places not only the hydrogenosomal AAC gene of *Neocallimastix* (*hdgaac*), but also the putative AACs from *Nyctotherus* with high reliability values (98/100) into a well-defined, monophyletic cluster of mitochondrial AACs (Fig. 3). Monophyly of the hydrogenosomal AAC of *Neocallimastix*

Table 3. Cardiolipin in anaerobic protists.

	Neocallimastix sp. L2	*Piromyces* sp. E2	*Nyctotherus ovalis*
Cardiolipin	–	–	+

Total lipids were extracted using the method of Bligh and Dyer (1959). Negative ion mass spectrometry was performed on a Sciex API 365 triple quadrupole mass spectrometer equipped with an electrospray ion source. The presence or absence of cardiolipin ions in the mass spectra was studied by analysis of the fragments produced by collision-induced dissociation of the putative cardiolipin ions. Mass spectra are available as *Supplementary material*.

and the mitochondrial AACs from the fungal–plant clade (cf. Löytynoja and Milinkovitch, 2001) is supported with reliability values of 89/99, respectively, whereas the AACs from *Nyctotherus* cluster with the AAC of the aerobic ciliate *Euplotes* and the mitochondrial AACs from the various animals. The clustering of hydrogenosomal and mitochondrial AACs of ciliates in the same branch is supported by the shared 'RRRLMM' motif (Fig. 1) and consistent with evidence from a phylogenetic analysis of the organelle's SSU rRNA genes (Akhmanova *et al*., 1998a; van Hoek *et al*., 2000).

After expression in *E. coli*, the protein encoded by the *hdgaac* gene of *Neocallimastix* sp. L2 facilitates the preferential import of ADP into the bacterial cells, as expected for a mitochondrial-type AAC (Tables 1 and 2; cf. Tjaden *et al*., 1998; Winkler and Neuhaus, 1999). As earlier studies have demonstrated that incubation of isolated hydrogenosomes of *Neocallimastix* with bongkrekic acid and carboxyatractylate, well-known inhibitors of mitochondrial AACs (Winkler and Neuhaus, 1999), causes inhibition of hydrogen formation by about 75% (Marvin-Sikkema *et al*., 1994), it must be concluded that the mitochondrial-type AAC described here fulfils a crucial function in the hydrogenosomal metabolism of *Neocallimastix*.

The gene encoding Hmp31, a mitochondrial-type carrier of unknown specificity in the hydrogenosomes of *T. vaginalis* (Dyall *et al*., 2000), does not cluster with any of the hydrogenosomal and mitochondrial AACs (Fig. 3). Nevertheless, Hmp31 has been postulated to function as the hydrogenosomal ADP/ATP carrier of *T. vaginalis* (Dyall *et al*., 2000). Notably, the absence of a veritable mitochondrial-type AAC is supported by the observation that atractyloside, a highly specific inhibitor of mitochondrial AACs, inhibits adenine nucleotide uptake by isolated hydrogenosomes of *Trichomonas foetus* only poorly (Cerkasov *et al*., 1978). Furthermore, we failed to amplify mitochondrial-type AAC homologues by PCR in both *T. vaginalis* and *T. gallinae.* Conversely, a highly conserved Hmp31 homologue could easily be recovered from *T. gallinae* (Fig. 1), but the PCR primers directed against Hmp31 failed to amplify any gene related to Hmp31 from genomic DNA of *Neocallimastix*, *Piromyces* and several aerobic and anaerobic ciliates (data not shown). Speculations as to whether trichomonad hydrogenosomes lack a mitochondrial-type AAC but possess an ancestral carrier with similar function can, of course, only be resolved by genome sequencing and direct experimentation.

Also, phylogenetic analyses of chaperonin cpn 60 from *T. vaginalis* support multiple, independent origins for hydrogenosomes. The chaperonin 60 of *T. vaginalis* clusters with cpn 60s from amitochondrial protists, providing further evidence that the *Parabasalia* belong to the early branching eukaryotes (Horner and Embley, 2001). In contrast, the cpn 60s of *Neocallimastix* sp. L2 and *Piromyces* sp. E2, an anaerobic, hydrogenosome-bearing close relative of *Neocallimastix* (cf. Akhmanova *et al*., 1998b; 1999; Hackstein *et al*., 1999; Brookman *et al*., 2000), cluster with high bootstrap support (85/95) with the homologous, mitochondrial chaperonins from aerobic yeasts and fungi (Fig. 8). The mitochondrial chaperonins 60 (HSP 60) from animals and aerobic protists occupy positions in the phylogenetic tree that are clearly distinct from both fungal and trichomonad branches (Fig. 8; cf. Horner and Embley, 2001). These data corroborate the conclusions drawn from the analysis of hydrogenosomal AACs and SSU rRNA genes.

In contrast to the molecular data, the ultrastructure of hydrogenosomes, in general, fails to provide clues for a mitochondrial ancestry. The hydrogenosomes of *Nyctotherus* are an exception because they exhibit a mitochondria-like morphology (Akhmanova *et al*., 1998a) and possess cardiolipin in their membranes like 'real' mitochondria (Table 3). The morphology of the hydrogenosomes of *Neocallimastix* sp. L2, *Piromyces* sp. E2 and *Trichomonas* spp., on the other hand, is rather different from that of 'textbook' mitochondria (Figs 5–7; Benchimol *et al*., 1996; 1997a). Moreover, they lack cardiolipin, a lipid that is believed to be essential for both the insertion of mitochondrial AACs into the inner membrane of mitochondria and their proper function (Hoffmann *et al*., 1994). We have shown here by cell fractionation (Fig. 4) and immunocytochemistry (Fig. 5) that the AAC of *Neocallimastix* is an integral component of the hydrogenosome membranes. The hydrogenosomal AAC protein is resistant towards extraction with 1 M KCl, excluding a peripheral or loose membrane association. Carbonate extraction, on the other hand, is able to release a minor but significant fraction of AAC (Fig. 4D). However, the lack of cardiolipin in the hydrogenosomal membranes of *Neocallimastix* is likely to reduce the stability of the AAC–membrane complexes, allowing the extraction of a larger fraction of AAC than in mitochondria that are treated in the same way.

The hydrogenosomes of *Neocallimastix* sp. L2 lack cristae and other differentiations characteristic of a mitochondrial inner membrane. Also, they lack a morphologically distinct mitochondrial-type outer membrane (Figs 5–7). The three-dimensional reconstruction from serial ultrathin sections of hydrogenosomes from zoospores revealed no principal differences from hydrogenosomes that had been isolated from hyphal homogenates by differential centrifugation (Figs 6 and 7; cf. Marvin-Sikkema *et al*., 1992; 1993a). Neither electron microscopy using cryofixation nor a systematic variation of the chemical fixation procedures (data not shown) provided any evidence for 'double' membranes that has been suggested

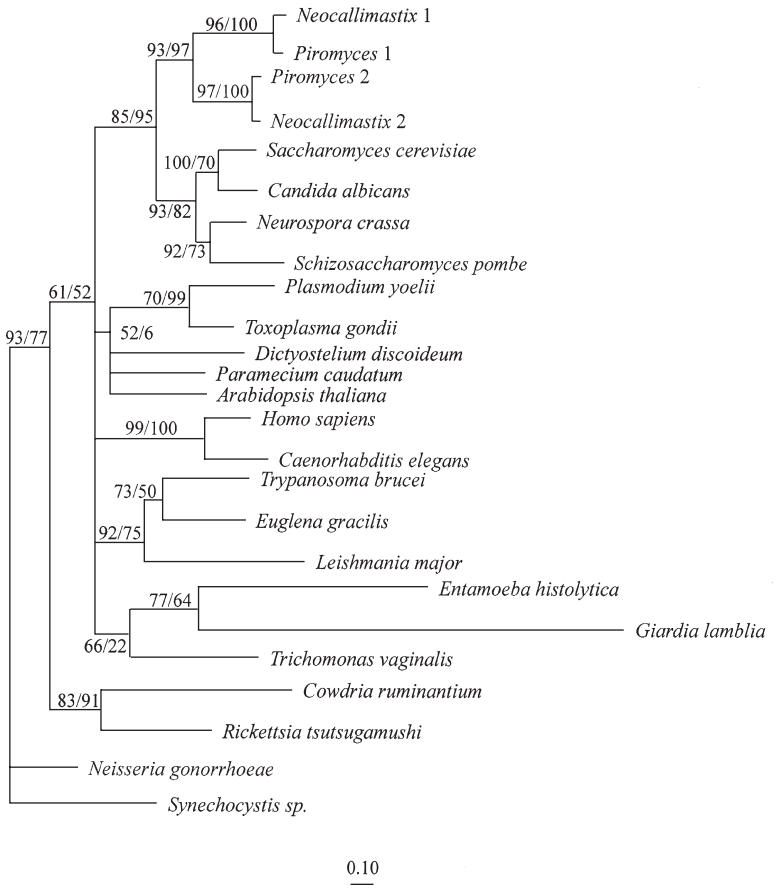

Fig. 8. Phylogenetic tree of HSP (cpn) 60. See Fig. 3 for methods. The HSP 60s of the anaerobic chytrids *Piromyces* sp. E2 (and *Neocallimastix* sp.) cluster with high bootstrap values with the mitochondrial HSP 60s of aerobic yeasts and fungi. The HSP 60 of the ciliate *Paramecium caudatum* and the parabasalian flagellate *Trichomonas vaginalis* belong to different clades.

by Benchimol *et al*. (1997a) and van der Giezen *et al*. (1997). Moreover, the ultrastructure of *Neocallimastix* hydrogenosomes in the studies of Marvin-Sikkema *et al*. (1992; 1993a,b), Brondijk *et al*. (1996), Hackstein *et al*. (2001) and Voncken *et al*. (2002) does not differ from that shown here. We assume that the 'double-membrane' appearance of certain sections through the hydrogenosomes in the studies of van der Giezen *et al*. (1997) and Benchimol *et al*. (1997a) is caused by a swelling of the organelles during the isolation and fixation procedure.

The lack of a double membrane does not necessarily provide arguments in favour of a microsomal (peroxisomal) origin for the hydrogenosomes of *Neocallimastix* as suggested earlier (Marvin-Sikkema *et al*., 1993b). Ultrastructural studies on the mitochondria of patients with hereditary mitochondrial diseases have shown that mitochondria can exhibit highly abnormal morphologies (Smeitink *et al*., 1989; Huizing *et al*., 1997; Frey and Mannella, 2000), and the lack of cardiolipin in mutant CHO cells can have dramatic consequences for the shape of mitochondria (Ohtsuka *et al*., 1993). The application of 'Occam's razor' argues strongly for a mitochondrial ancestry of the hydrogenosomes of chytrids, notwithstanding their unique morphology and their peculiar biochemistry (this paper; Hackstein *et al*., 1999). As discussed above, the phylogenetic analysis of AACs, HSP 60s and the SSU rRNA genes of certain anaerobic ciliates suggested a recent common ancestry of chytrid and ciliate hydrogenosomes with the mitochondria of their aerobic relatives. As the molecular data available for the hydrogenosomes of *T. vaginalis* and *T. gallinae* argue for an ancestry that is distinct from both the ciliate's and the chytrid's (Fig. 8; cf. Horner and Embley, 2001), we postulate that the hydrogenosomes of the parabasalia, anaerobic chytrids and anaerobic ciliates are varieties of 'anaerobic' mitochondria that arose independently. In line with this reasoning, the hydrogenosomes of *Trichomonas* might represent the oldest branch that might eventually be traced back to the protomitochondrial ancestor.

Experimental procedures

Organisms and growth conditions

Neocallimastix sp. L2 was cultured axenically under anoxic conditions in M2 medium, supplemented with 20 mM fructose, at 39°C (Teunissen *et al*., 1991; Marvin-Sikkema *et al*., 1992). The aerobic ciliate *Tetrahymena thermophila* was cultured axenically in a medium containing proteose peptone, yeast extract, glucose and ferrous sulphate/chelate solution (Sigma F 0518). *Euplotes minuta*, a generous gift from

Professor H.-D. Görtz, Stuttgart (Germany), was grown in artificial sea water and fed with *E. coli.* The anaerobic heterotrichous ciliates of the *N. ovalis* cluster were isolated by electromigration from the hindgut of cockroaches as described earlier (van Hoek *et al*., 1998, 1999). *Trichomonas gallinae* was cultured in Diamond's trypticase–yeast extract–maltose medium (TYM; cf. Vanacova *et al*., 2001). *T. vaginalis* was a generous gift from Professor Dr H. Mehlhorn, Düsseldorf (Germany).

Isolation of genomic DNA and messenger RNA

Genomic DNA from the chytrids was prepared according to the protocol of Brownlee (1994). Protozoan DNAs were isolated using a phenol–chloroform protocol. Total RNA from *Neocallimastix* sp. L2 was prepared by the guanidinium chloride method (Sambrook *et al*., 1989). Poly(A)$^+$ RNA was isolated with the Pharmacia QuickPrep mRNA purification kit.

Isolation of AACs, Hmp31 homologues and HSP 60

Adaptor-ligated cDNA was prepared from poly(A)$^+$ RNA using the Clontech Marathon cDNA isolation kit. Oligonucleotide primers based on highly conserved amino acid regions of ADP/ATP carriers and DNA PCR were used to amplify DNA from adapter-ligated cDNA. A full-length cDNA (constructed from one nearly complete 5′ RACE clone and the corresponding 3′ RACE clone) was isolated by the RACE procedure as described in the protocol of the Clontech Marathon cDNA isolation kit. Sequencing was performed with the ABI Prism model 310 automatic sequencer, using a rhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems).

AACs from ciliates and *Toxoplasma gondii* were isolated using PCR on total DNA with primers directed against conserved regions of known mitochondrial AACs or ESTs, respectively, and subsequently cloned in pGEM-T easy (Promega). Attempts to isolate AAC homologues from *T. vaginalis* and *T. gallinae* using these primers were not successful. On the basis of primers described by Dyall *et al*. (2000), an Hmp31 homologue could be amplified and cloned from *T. gallinae*. The HSP 60 gene of *Piromyces* was recovered by PCR from a lambda ZAP II library described earlier (Akhmanova *et al*., 1998b; 1999). The sequences of the primers used in this study and the alignments are available on request.

Northern and Southern blotting

Poly(A)$^+$ RNA (5 μg) from *Neocallimastix* sp. L2 was size fractionated on a 1% agarose–formaldehyde gel. Genomic DNA (10 μg) was extracted from *Neocallimastix* sp. L2, digested with the various restriction enzymes and separated on 0.7% agarose gels. The gels were blotted to Hybond N+ membranes (Amersham). The *hdgaac* probe was labelled with [α-^{32}P]-dATP using PCR. Hybridization was performed in 0.5 M sodium phosphate buffer, pH 7.0, 7% SDS, 1% BSA and 1 mM EDTA at 60°C. The membrane was washed stringently with 50 mM sodium phosphate buffer, pH 7.0, and 0.5% SDS at 60°C.

Functional expression in E. coli

Expression of the *hdgaac* of *Neocallimastix* sp. L2 in *E. coli* was performed as described by Tjaden *et al*. (1998). The uptake of [α-^{32}P]-ADP in competition with various effectors was measured in the presence of the various effectors that were applied at a concentration of 300 μM. [α-^{32}P]-ADP was present at a concentration of 100 μM. Uptake was carried out for 4 min and stopped by rapid filtration.

Sequence analysis

Sequence alignments were created with T_COFFEE (Notredame *et al*., 2000) from a representative set of sequences of the mitochondrial carrier family or the HSP 60 family respectively. Phylogenetic trees, based on the positions present in all aligned sequences, were calculated with PUZZLE (Strimmer and von Haeseler, 1996) using eight gamma-distributed rate categories and the JTT model of protein sequence evolution (Jones *et al*., 1992). Alternatively, distance-based phylogenies were calculated using neighbour joining (Saitou and Nei, 1987). For both sets of sequences, all the tree partitions, which were resolved with PUZZLE, were consistently predicted by neighbour joining. The (partial) *T. gallinae* sequence is 92% identical to the Hmp31 of *T. vaginalis.* It was omitted from the calculations because it would reduce the number of aligned positions that could be used for the tree.

The GenBank identifiers or, if available, SWISSPROT names of the mitochondrial carrier family are *N. ovalis* BD AAC1: AF480921; *N. ovalis* PAB (AAC1): AF480919; *N. ovalis* BD AAC2: AF480922; *N. ovalis* PAB (AAC2): AF480920; *Neocallimastix* sp. L2 hdgaac: AF340168; *E. minuta*: AF480923; *Leishmania major*: g7018577, *T. brucei*: g3220183, *S. cerevisiae* (AAC2): ADT2_YEAST, *S. cerevisiae* (AAC3): ADT3_YEAST, *S. cerevisiae* (AAC1): ADT1_YEAST, *Kluyveromyces lactis*: ADT_KLULA, *Neurospora crassa*: ADT_NEUCR, *Schizosaccharomyces pombe*: ADT_SCHPO, *Chlamydomonas reinhardtii*: ADT_CHLRE, *Triticum turgidum*: ADT1_WHEAT, *S. cerevisiae* (YGH2): YGH2_YEAST, *Homo sapiens* (GDC): GDC_HUMAN, *S. cerevisiae* (YPR011c): g939745, *T. vaginalis*: g6746567, *Chlorella kessleri*: ADT_CHLKE, *Drosophila melanogaster*: ADT_DROME, *H. sapiens* (AAC3): ADT3_HUMAN, *H. sapiens* (AAC2): ADT2_HUMAN, *H. sapiens* (AAC1): ADT1_HUMAN, *Dictyostelium discoideum*: g3885438, *T. gondii*: g13445087, *S. cerevisiae* (TXTP): TXTP_YEAST, *H. sapiens* (TXTP): TXTP_HUMAN, *S. cerevisiae* (MPCP): MPCP_YEAST, *H. sapiens* (MPCP): MPCP_HUMAN, *H. sapiens* (M2OM): M2OM_HUMAN, *H. sapiens* (MCAT): MCAT_HUMAN, *Zea mays* (BT1): BT1_MAIZE, *S. cerevisiae* (FLX1): FLX1_YEAST, *S. cerevisiae* (YGR069W): g6321533, *H. sapiens* (DIC): g6179584.

The GenBank identifiers of the HSP60 sequences are *Neocallimastix* 1: AY078243, *Neocallimastix* 2: AY078244, *Piromyces* 1: AF426026, *Piromyces* 2: AY078242, *N. crassa*: g7800840, *S. pombe*: g1346314, *Candida albicans*: g3552009, *S. cerevisiae*: g123579, *H. sapiens*: g190127, *Arabidopsis thaliana*: g116229, *D. discoideum*: g1621639, *T. gondii*: g5052052, *Plasmodium yoelii*: g3885995, *L. major*: g3023477, *Euglena gracilis*: g2493645, *T. brucei*: g249365,

Entamoeba histolytica: g2564749, *T. vaginalis*: g1755053, *Cowdria ruminantium*: g1345759, *Neisseria gonorrhoeae*: g2119968, *Synechocystis*: g2506274, *Paramecium caudatum*: g13359321.

Subcellular fractionation

Hydrogenosomal and cytosolic fractions were obtained by differential centrifugation, using the protocol of Marvin-Sikkema *et al*. (1993a) with minor modifications. The isolation procedure was monitored by measurements of the activities of hexokinase, hydrogenase and adenylate kinase (cf. Table 1 in Voncken *et al*., 2002) and, once, by electron microscopy (see also Fig. 1 in Marvin-Sikkema *et al*., 1993a). For the isolation of hydrogenosomal membranes, the hydrogenosomal fraction was resuspended in 100 mM Tris-HCl buffer, pH 7.5. The hydrogenosomes were lysed by repeated freezing and thawing and subsequently centrifuged (233 000 *g* for 60 min at 4°C). The pellet, consisting of the hydrogenosomal membranes, was extracted twice with 1 M KCl or 0.1 M sodium carbonate (pH 11.5) for 60 min on ice. Subsequent centrifugation (233 000 *g* for 60 min at 4°C) resulted in a supernatant containing membrane-associated proteins and a pellet that contained integral membrane proteins. All extraction steps were performed at 4°C in the presence of the Complete protease inhibitor mix (Boehringer Mannheim). Equal portions of all fractions were separated on 12.5% polyacrylamide gels containing 0.1% SDS (SDS–PAGE) and blotted to a PVDF membrane by semi-dry transfer (Bio-Rad) according to the manufacturer's protocol. A polyclonal antiserum, directed against the mitochondrial ADP/ATP carrier (AAC) of the yeast *S. cerevisiae*, was used to identify the putative hydrogenosomal AAC using goat anti-rabbit antibodies conjugated to peroxidase (Boehringer Mannheim).

For the proteinase K protection experiments, isolated hydrogenosomes (about 0.4 mg of protein) were incubated anaerobically in a medium consisting of 0.5 ml of 20 mM HEPES, pH 7.4, 250 mM sucrose and 2 mM dithiothreitol (DTT). Proteinase K (50 μg per 0.5 ml assay volume) or detergent (0.1% Triton X-100 and 0.1% deoxycholate) was added when indicated (see Fig. 4b). After 30 min incubation on ice, the proteinase K treatment was terminated by the addition of 0.5 ml of 15% (w/v) TCA. Aliquots of each incubation were separated on a 12.5% SDS–polyacrylamide gel, blotted to a PVDF membrane and probed with the AAC antiserum.

For the N-terminal amino acid sequencing, the cross-reacting protein band was cut out from the PVDF membrane and subjected to microsequencing (Applied Biosystems, Model 477A).

Cardiolipin analysis

Cardiolipins were analysed in a total lipid extract, which was prepared according to the method described by Bligh and Dyer (1959). Negative ion mass spectrometry was performed on a Sciex API 365 triple quadrupole mass spectrometer equipped with an electrospray ion source. The presence or absence of cardiolipin ions in the mass spectra was studied by analysis of the fragments produced by collision-induced dissociation of the putative cardiolipin ions.

Electron microscopy, immunogold labelling and three-dimensional reconstruction

Electron microscopy and immunocytochemistry with the *S. cerevisiae* anti-AAC was performed as described by Marvin-Sikkema *et al*. (1993b). For the osmotic treatment, hydrogenosomes were isolated as described above and resuspended in (anoxic) isolation buffer (20 mM HEPES, pH 7.4, 2 mM DTT) containing 0.0, 0.1, 0.25, 0.5 or 1.0 M sucrose and incubated on ice for 30 min. The hydrogenosomes were prefixed by the addition of glutaraldehyde (0.1% final concentration). After 15 min, the hydrogenosomes were collected by centrifugation and post-fixed in 2.5% glutaraldehyde for 2 h on ice.

For a three-dimensional reconstruction of the hydrogenosomes, fixed and Epon-embedded zoospores of *Neocallimastix* sp. L2 were subjected to serial sectioning. Sixteen serial electron micrographs were mounted on a digitizer tablet. The relevant contours were traced manually and stored in a database using the three-dimensional reconstruction software TDR-3DBASE (Verbeek *et al*., 1995). For each section, two reference points were included and used to realign the contours to a consistent three-dimensional stack of contours. The contour model was used to generate a voxel model of cells containing the hydrogenosomes (Verbeek, 1999 and references therein). TDR-3DBASE was developed for the MS Windows (9×, NT) user interface. Here, a Summagraphics Bitpad Two was used for the input of the contours (Verbeek, 1999). Visualization of the volume model was accomplished with a SUN UltraSPARC 10 workstation.

Data deposition

The sequence data reported in this paper have been deposited in the EMBL/GenBank databases. Accession numbers are: hdgaac:, AF340168; Tgaac, AF343580; PAB AAC1, AF480919; PAB AAC2, AF480920; BD AAC1, AF480921; BD AAC2, AF480922; euplotesaac, AF480923; E2hsp60, AF426026; E2HSP60-2, AY078242; L2HSP60-1, AY078243; L2HSP60-2, AY078244.

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Supplementary material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/mole/mole2959/mmi2959sm.htm

Fig. S1. Mass spectrum for Piromyces (negative for cardiolipin).
Fig. S2. Mass spectrum for Nyctotherus (positive for cardiolipin).
Fig. S3. Product spectrum of hydrogenosomal cardiolipin.

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